Journal of Microbiological Methods 87 (2011) 133–138

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Review

Sporulation studies in *Clostridium difficile*

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ARTICLE INFO

Article history:
Received 4 April 2011
Received in revised form 18 July 2011
Accepted 21 July 2011
Available online 16 August 2011

Keywords:
Clostridium difficile
Spore
Sporulation
BI/NAP1/027
Hypervirulent

ABSTRACT

Clostridium difficile is a leading cause of healthcare-associated diarrhoea. In recent years, certain *C. difficile* types have become highly represented among clinical isolates and are associated with outbreaks of increased disease severity, higher relapse rates and an expanded repertoire of antibiotic resistance. Endospores, produced during sporulation, play a pivotal role in infection and disease transmission and it has been suggested in the literature that these so-called 'hypervirulent' *C. difficile* types are more prolific in terms of sporulation *in vitro*. However, work in our laboratory has provided evidence to the contrary suggesting that although there is significant strain-to-strain variation in *C. difficile* sporulation characteristics this variation does not appear to be type-associated. On analysis of the literature, it is apparent that the methods used to quantify sporulation in previous studies have varied greatly and sample sizes have remained small. The conflicting data in the literature may, therefore, not necessarily be generally representative of *C. difficile* sporulation. Instead, these inconsistencies may reflect differences in the experimental design of each study. In this review, the need for further investigations of *C. difficile* sporulation rates is highlighted. Specifically, the advantages and disadvantages of the different experimental approaches previously used are discussed and a standard set of principles for measuring *C. difficile* sporulation in the future is proposed.

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Contents

1. Introduction

Clostridium difficile is a leading cause of hospital-acquired diarrhoea and a major burden to healthcare services worldwide. In recent years, *C. difficile* infection (CDI) has led to patient isolation, ward closures and, in some cases, hospital closure. In the United States of America alone, CDI is estimated to affect over 500,000 people each year and is estimated to cost the healthcare system over $3 billion per year (Cloud and Kelly, 2007; Rupnik et al., 2009). Endospores, produced during sporulation, are pivotal to disease transmission. These spores are able to resist a variety of physical and chemical agents (Setlow, 2007), allowing for *C. difficile* persistence on surfaces in the healthcare setting. The subsequent inadvertent ingestion of contaminated material leads to infection or re-infection of co-habiting

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doi:10.1016/j.mimet.2011.07.017

individuals (Gerding et al., 2008; Riggs et al., 2007). On reaching the anaerobic environment of the gut, the ingested spores presumably germinate to form toxin-producing vegetative cells and, in susceptible individuals, cause diarrhoeal disease.

The emergence of *C. difficile* strains belonging to restriction endonuclease type BI, North American pulsed-field type 1 (NAP1) and PCR-ribotype 027 (BI/NAP1/027) has contributed to the problem of *C. difficile* incidence (Kuijper et al., 2007; Pépin et al., 2004). These so-called 'hypervirulent' types were in the past isolated infrequently from patients suffering from CDI but have recently become highly represented among such clinical isolates. In addition, emerging strains belonging to PCR-ribotype 017 and 078, that are also associated with severe disease, have been isolated in parts of Asia and Europe (Drudy et al., 2007; Kim et al., 2008). Unsurprisingly, there is a widespread interest in understanding the underlying factors that have led to the emergence of hypervirulent *C. difficile* strains, such as those of the BI/NAP1/027 type.

As the spore form is considered to be the vehicle for the transmission of CDI, knowledge of how sporulation characteristics vary among strains is important. A number of recent studies have concluded that 'epidemic' and hypervirulent *C. difficile* types are more prolific in terms of sporulation *in vitro* than non-epidemic types (Akerlund et al., 2008; Fawley et al., 2007; Merrigan et al., 2010; Vohra and Poxton, 2011; Wilcox and Fawley, 2000). To the contrary, data from our laboratory suggest that although there is substantial variation in *C. difficile* sporulation characteristics this variation does not appear type-associated (Burns et al., 2010a). Importantly, four of the above studies included the same *C. difficile* strain in their analysis but the conclusions as to its sporulation proficiency were not consistent (Akerlund et al., 2008; Burns et al., 2010a; Merrigan et al., 2010; Vohra and Poxton, 2011). Interestingly, the methods used to quantify sporulation have varied significantly from study to study and, currently, there is no accepted 'gold standard' procedure for quantifying *C. difficile* sporulation. For this reason, the differences in the findings of these studies may reflect the experimental approaches employed and, while the overall findings of each study are important, some of these data may have been misinterpreted based on small sample sizes and methods that cannot quantify sporulation independently of other important properties.

The intention of this article is to compare the methods previously used to assess *C. difficile* sporulation characteristics and discuss how varying the experimental design may lead to a different interpretation of the data. Furthermore, we propose a standard set of procedures to be considered when quantifying *C. difficile* sporulation, which may be generally deployed in the future to better understand the characteristics of emerging, clinically important *C. difficile* types.

2. Quantifying *C. difficile* sporulation

When attempting to gain a quantitative measurement of *C. difficile* sporulation, it is important to ensure that the resultant data are representative of sporulation alone. Accordingly, sporulation must be measured independently of factors such as vegetative cell growth, the death of non-sporulating cells, spore resistance properties, spore germination and the subsequent vegetative cell outgrowth. Recently, a number of different approaches have been used to quantify *C. difficile* sporulation. The advantages and disadvantages of each approach are discussed below.

2.1. Measuring sporulation based on colony-forming units

Perhaps the most widely used method for enumerating *C. difficile* spores involves measuring colony-forming units (CFU) on agar plates after a stress treatment such as heat or ethanol shock (Buckley et al., 2011; Goulding et al., 2009; Kamiya et al., 1992; Kuehne et al., 2010; Lyras et al., 2009; Poxton et al., 2001; Sambol et al., 2001). *C. difficile* spores are able to survive incubation at 60–80 °C for 10–30 min or incubation with up to 100% ethanol for an extended period of time (Setlow, 2006; Setlow, 2007). The rationale of this approach is that only spores will survive the treatment and the colonies recovered on plates are, therefore, representative of spores within the population. By analysing the change in stress-resistant CFU over a defined period of time it is then possible to measure the development of spores within a liquid culture and consequently estimate the rate at which *C. difficile* forms spores (Burns, et al., 2010a). However, the CFU obtained after exposure to heat or ethanol shock represent vegetative cells that have (i) completed sporulation; (ii) resisted the heat/chemical treatment; (iii) completed germination; and (iv) returned to vegetative cell growth. When studying different strains of *C. difficile*, or even different mutants derived from the same *C. difficile* parental strain, it is important to consider that there will likely be variation in characteristics such as cell growth rate, spore resistance properties and germination proficiency. Indeed, our recent analysis of *C. difficile* clinical isolates indicated that there was significant variation in the proportion of spores that form colonies on nutrient-rich medium following heat treatment (Burns, et al., 2010a). Consequently, the number of observed stress-resistant CFU is not directly comparable to the number of spores within the same culture and measurements of CFU should always be combined with another procedure, such as analysis by phase-contrast microscopy (see Section 2.2), in order to accurately observe spore numbers.

As spores must complete germination in order to form colonies, it is also important that CFU are enumerated on a medium that is appropriately supplemented to support efficient germination. This is often achieved using the bile salt taurocholate which, when added to rich medium, has been shown to result in approximately 10^5-fold higher recovery of spores when compared to medium lacking taurocholate (Sorg and Sonenshein, 2008; Wilson et al., 1982). A recent study analysed the spore titres of a number of *C. difficile* strains by enumerating ethanol-resistant CFU on blood agar plates (Vohra and Poxton, 2011). However, the medium was not supplemented with taurocholate and the highest number of CFU recovered from the five tested strains was an average of 443 CFU/ml. The proportion of spores recovered for each strain would likely have been significantly higher using medium supplemented with taurocholate. Therefore, the differences observed among strains in this study were small, making statistical validation of the data problematic.

2.2. Counting *C. difficile* spores using phase-contrast microscopy

A simple means to measure sporulation independently of other characteristics is to enumerate spores using phase-contrast microscopy. Such an approach allows for a measurement of the exact number of spores in a population as well as, for comparative means, the number of vegetative cells present in the same population. Phase-bright spores are generally easily distinguished from vegetative cells when observed by microscopy, but a number of studies have reported using specific staining procedures to further ease this distinction (Fawley et al., 2007; Underwood et al., 2009). An example of such a method is the Schaeffer–Fulton endospore stain (Mormak and Casida, 1985). This involves a primary malachite green stain which traverses the spore coat on heat treatment but is retained within the coat after cooling. A safranin counter-stain is then taken up by vegetative cells but not by spores, resulting in spores appearing as green and vegetative cells appearing as pink when observed by microscopy. The ideal way to then count spores or cells in this manner is to use a specialised counting chamber such as a haemocytometer, and examples of using this approach to count *C. difficile* spores have been described (Akerlund et al., 2006; Burns et al., 2010a; Burns et al., 2010b).

Despite the benefit of using counting chambers to measure the number of spores within a suspension, some recent studies have expressed sporulation differently. In a study by Merrigan and co-workers,

where BI/NAP1/027 isolates were suggested to be more proficient in terms of sporulation, the numbers of spores per field at 100× magnification were enumerated (Merrigan et al., 2010). Such a procedure allows for an accurate comparison of spore numbers among different cultures, although it must be noted that four of the eight strains tested returned a mean count of <5 spores per field when 10 fields were observed. Consequently, it could be argued that these counts may not be satisfy the necessary criteria for measurements of statistical significance and their findings may require further verification.

2.3. Expressing sporulation as the percentage of spores within a population

A study by Akerlund and co-workers has also concluded that BI/NAP1/027 strains exhibit higher sporulation rates than non-epidemic strains (Akerlund et al., 2008). In this study, although both spores and vegetative cells were enumerated using a counting chamber, the sporulation rate was expressed as the ratio of spores to vegetative cells within the population after 48 h incubation, an approach other studies have used in a similar manner (Fawley et al., 2007; Underwood et al., 2009; Wilcox and Fawley, 2000). These measurements are important, as the raw data can provide information on both the spore titre and the fate of non-sporulating vegetative cells. However, when only a spore-to-cell ratio is reported, it is not possible to distinguish between these two factors, or indeed the generic growth characteristics of the isolates studied. As an example, if two *C. difficile* strains were found to form spores in equal numbers over a defined time period and the same proportion of vegetative cells initiated sporulation in each strain, the subsequent sporulation rates of both strains should be expressed as equal. However, following the initiation of sporulation, if more non-sporulating vegetative cells were to survive in one strain than in another, a higher vegetative cell count would then lower the percentage of spores in the population when the suspensions are analysed by microscopy. As a result, two strains forming identical numbers of spores may be described to have different sporulation characteristics. For these reasons, by only reporting the percentage of spores present within a population, it may not be possible to make precise assumptions on sporulation rates. A more comprehensive study might combine such an analysis with the actual number of spores counted in the suspension. Accordingly, an accurate argument could then be made regarding both sporulation proficiency and the fate of vegetative cells that do not enter the sporulation process.

2.4. Concluding remarks

Clearly, the precise methods recently used to quantify *C. difficile* sporulation have varied greatly from study to study. Based on the limitations of each approach described above, there is a risk that, while interesting observations have been made, these observations are not readily comparable. Furthermore, some of these data may not be truly representative of differences in sporulation rates among the *C. difficile* strains tested. We feel that phase-contrast microscopy analysis is essential when enumerating *C. difficile* spore titres and this should be based on either the use of a counting chamber or by comparing the number of spores observed per field of view. Furthermore, regardless of what further analysis is undertaken, the spore count should always be supplied to avoid misinterpretations of data by either authors or readers.

3. Further experimental procedures

When analysing *C. difficile* sporulation, the procedures used to enumerate spores are only one aspect to consider. Differences in the basic experimental setup among studies may affect the results even when the same *C. difficile* strains are evaluated. To date, four independent studies have analysed the *C. difficile* reference strain VPI 10463. However, the conclusions of these studies were not consistent regarding the sporulation proficiency of this strain (Akerlund et al., 2008; Burns et al., 2010a; Merrigan et al., 2010; Vohra and Poxton, 2011). Important variations in the experimental setups of these studies are discussed below in an effort to understand how these conflicting reports may have arisen.

3.1. Growth conditions

In spore-forming organisms such as *Bacillus subtilis*, it is well established that sporulation efficiency varies greatly depending on the choice of growth medium (Errington, 1993). Therefore, it stands to reason that altering the growth medium may also affect *C. difficile* sporulation.

Perhaps the most widely used medium for inducing *C. difficile* sporulation is brain-heart infusion (BHI) supplemented with L-cysteine and yeast extract (BHIS), and a number of studies have used either this medium, or a non-supplemented BHI, to measure *C. difficile* sporulation and to prepare spores for subsequent analyses of spore germination (Burns et al., 2010a,b; Merrigan et al., 2010; Sorg and Sonenshein, 2008, 2009, 2010; Underwood et al., 2009). Use of a different medium was described in a study by Akerlund et al., where *C. difficile* strains were cultivated in a peptone-yeast medium with added cysteine (Akerlund et al., 2006). However, in later work by the same group, cysteine was not added to the peptone-yeast sporulation medium (Akerlund, et al., 2008). The decision not to include cysteine in this sporulation medium could be explained based on reported evidence that cysteine may inhibit *C. difficile* toxin production when added to late-exponential-phase cultures (Karlsson et al., 2008), as toxin production was also analysed in this study (Akerlund et al., 2008). Finally, two other studies have used a faecal emulsion to prepare *C. difficile* spores, an approach that is particularly interesting as these conditions will likely mimic the natural *in vivo* environment of *C. difficile* (Fawley et al., 2007; Wilcox and Fawley, 2000).

Our knowledge of *C. difficile* sporulation mechanisms remains limited, with few studies reported to date (Heap et al., 2007; Underwood, et al., 2009). For this reason, it is not clear exactly what the desirable components of a sporulation medium are. Likewise, it is not clear what components of currently used sporulation media may be having a limiting effect on sporulation. Studies describing in detail the effects of different media on *C. difficile* sporulation would, therefore, be welcomed in the future.

3.2. Examining sporulation over time

To gain a detailed insight into *C. difficile* sporulation rates, it is important to measure spore formation over multiple time-points. Such an analysis can allow for a distinction of (i) when sporulation is initiated; and (ii) the total number of spores that a particular strain produces under the growth conditions employed. Ideally, to measure the total number of spores produced, sporulation should be measured every 24 h until the number of spores reaches a steady state. The exact period of time required for completion of the sporulation process in *C. difficile* is still not clear, although data from our laboratory suggest that this process is generally complete after 120 h (Burns et al., 2010a). However, we noted that not all of the strains tested in our study formed a maximal number of spores within 120 h. Consequently, the substantial variation in sporulation characteristics observed among strains may, in some cases, require a longer incubation in order to observe the total number of spores produced.

Other studies of *C. difficile* sporulation rates have been limited to a time-course of between 24 h and 72 h (Akerlund et al., 2008; Fawley et al., 2007; Merrigan et al., 2010; Vohra and Poxton, 2011; Wilcox and Fawley, 2000). We observed that a low spore titre at 24 h in comparison to other isolates did not necessarily correlate to a low

spore titre at 120 h. To the contrary, while *C. difficile* VPI 10463 appeared to form few spores after 24 h, a high number of spores was observed after 120 h (Burns, et al., 2010a). This observation is interesting as other studies have suggested that VPI 10463 is a strain with a low sporulation frequency (Akerlund, et al., 2008; Merrigan, et al., 2010; Vohra and Poxton, 2011). However, when taking into account our data, the previous studies may not have observed sporulation for a sufficient time to obtain a total spore count. As a result, it may be inaccurate to describe VPI 10463 as a low-sporulating *C. difficile* strain. On the other hand, when compared to other individual strains, a high number of spores observed in our study after 24 h did not always result in high numbers of spores after 120 h when compared to other strains (Burns, et al., 2010a). This suggests that in a case where a strain initiates sporulation particularly early, such a strain may not necessarily form a higher number of spores than a strain which initiates sporulation at a later stage.

3.3. Controls

When studying *C. difficile* sporulation, three important controls are required. First, it should always be considered that generic growth differences may exist among different *C. difficile* strains. Consequently, diverse spore titres may not necessarily be a direct result of sporulation *per se* but instead a consequence of growth. By analysing the change in optical density at 600 nm (OD_{600}) growth differences, where appropriate, can be excluded when interpreting subsequent experiments. The majority of recent studies have included such measurements (Akerlund et al., 2008; Burns et al., 2010a, 2010b; Merrigan et al., 2010; Underwood et al., 2009; Vohra and Poxton, 2011), although it must be noted that some similar studies have elected not to include this analysis (Fawley, et al., 2007; Wilcox and Fawley, 2000).

Second, if sporulation is being observed over a defined time-period, it is important to confirm that as few spores as possible are present in the sporulation medium at 0 h. Excessive carryover of spores from previous passages would suggest that sporulation has been initiated within the culture prior to the start of the experiment. This may in turn affect the rate of subsequent sporulation when compared to a culture where no spores are present at 0 h and could, therefore, lead to a bias among spore counts. Only one other study outside of our laboratory has included a measurement of sporulation at 0 h (Vohra and Poxton, 2011) and we suggest that this is an important issue to be considered when analysing *C. difficile* sporulation in the future. To ensure minimal spores are present at 0 h, strains can be repeatedly sub-cultured on solid media prior to starting the experiment. A starter culture can then be prepared with a 1% inoculum of an overnight culture and incubated until an OD_{600} of between 0.2 and 0.5 is reached. Finally, the sporulation medium is inoculated with a 1% inoculum of this starter culture (Burns et al., 2010a).

The third fundamental control required is a sporulation-negative control. This is especially important when analysing CFU after stress treatments such as heat and ethanol as, even though vegetative cells would likely be killed by the treatment, simple errors in experimental technique could lead to erroneous CFU due to (i) contamination of solutions used in serial dilutions; (ii) contamination when plating; or (iii) incomplete heat/ethanol treatment. The ideal *C. difficile* sporulation-negative control is a *spo0A* mutant, where the master regulator of sporulation has been inactivated (Heap et al., 2007), and this strain is available upon request from our laboratory. Alternatively, a culture confirmed to be free of spores by microscopy, following repeated sub-culture with appropriate media, can control for errors in experimental technique when analysing CFU. However, this latter control can only identify technical errors during stress treatments and not the contamination of sporulation cultures at the beginning of the study. In that respect, a *spo0A* control is the more appealing option. Only one other study outside of our laboratory has used a negative control when measuring *C. difficile* sporulation (Underwood et al., 2009). Based upon this evidence, the lack of appropriate controls in a number of previous studies (Akerlund et al., 2008; Fawley et al., 2007; Merrigan et al., 2010; Vohra and Poxton, 2011; Wheeldon et al., in press; Wilcox and Fawley, 2000) may perhaps make it difficult to validate the results.

3.4. Sample sizes

When assessing the characteristics of a particular group of strains, such as the *C. difficile* BI/NAP1/027 type, it is important to study as many representatives of that group as is possible in order to improve confidence in the data. Our recent analysis of *C. difficile* sporulation included seven BI/NAP1/027 strains, including isolates from both North America and Europe to minimize the risk of sampling clonal strains, and we observed significant variation in sporulation proficiency within the BI/NAP1/027 group (Burns et al., 2010a). This diversity within one group of seven *C. difficile* strains suggests that, in order to accurately determine variation in sporulation between different groups, an appropriate sample size will be much larger than seven strains. However, while the sample used in our study was not particularly large, this does represent the largest sample size used to-date in such a study. Other reports of *C. difficile* sporulation rates have been limited to as few as one representative strain of each type (Fawley et al., 2007; Vohra and Poxton, 2011; Wilcox and Fawley, 2000). A small sample size of this nature is clearly appropriate for initial investigations, such as the study by Wilcox and Fawley (2000), but the continued use of small sample sizes may be contributing to the conflicting evidence that is currently present in the literature. For this reason, it seems obvious that all future *C. difficile* strain comparisons, not only those studies of sporulation rates, should be based on a much larger group of strains. Furthermore, the sporulation diversity previously observed within the BI/NAP1/027 type suggests that it may be incorrect to assume that all strains of this type have similar characteristics.

4. Conclusions and future directions

The spore form of *C. difficile* is crucial for disease transmission in healthcare environments. Spores can contaminate the surroundings of patients with CDI and persist for extensive periods of time. Strains of *C. difficile* that are found to have increased sporulation proficiencies are, therefore, of particular concern. There have been reports that 'hypervirulent' types of *C. difficile* can produce more toxin in the laboratory (Warny et al., 2005) and recently a number of studies have concluded that these emerging *C. difficile* types are more prolific in terms of sporulation *in vitro* (Akerlund et al., 2008; Fawley et al., 2007; Merrigan et al., 2010; Vohra and Poxton, 2011; Wilcox and Fawley, 2000). Given the importance of spores for disease transmission, it is tempting to assume that this reported phenomenon is correct. However, work in our laboratory has since provided credible evidence to the contrary suggesting that the strain-to-strain variation in *C. difficile* sporulation characteristics is not type-associated (Burns et al., 2010a). Therefore, it can be argued that the current literature contains insufficient evidence to conclude that epidemic *C. difficile* types form spores with greater efficiency compared to other types and this may be due in part to the variety of techniques used to assess sporulation characteristics in previous investigations.

This review has examined the methods used in recent studies to analyse sporulation, discussing the advantages and disadvantages of each approach. Using the experimental designs described in a number of studies, accurate judgements may not have been made on the sporulation proficiencies of the tested *C. difficile* strains. Consequently, the sporulation rates of emerging strains of *C. difficile* may not in fact differ to non-epidemic types. If this is the case, readers who are not

familiar with techniques used to quantify sporulation may be misled by the literature that is currently available. As a result, and in an effort to increase the clarity of future studies, we now propose a set of standard principles to be considered when analysing *C. difficile* sporulation. In Fig. 1, we illustrate an experimental design that is capable of distinguishing between factors such as (i) vegetative cell growth; (ii) sporulation rate; (iii) total sporulation; (iv) the fate of non-sporulating vegetative cells; (v) spore stress resistance; and (vi) spore germination and outgrowth.

By measuring sporulation over a period of at least 120 h it is possible to obtain an accurate account of the total number of spores produced and by taking measurements at multiple time-points the rate of sporulation can be expressed more precisely. We suggest that exact spore titres should be enumerated using phase-contrast microscopy and that this measurement should always be present when describing spore numbers. Additional assessments of sporulation may involve the analysis of stress-resistant CFU and the proportion of spores to vegetative cells within a population at defined time-points. We consider that suitable controls are mandatory when measuring sporulation. It is important that growth differences among strains are excluded before data on spore titres can be interpreted and, while particularly important when analysing CFU after stress treatments, the principle of ensuring no spores are present at 0 h also applies to studies where sporulation is enumerated in a different manner. In addition, any future report of *C. difficile* sporulation characteristics must include a sporulation-negative control in order to gain confidence in the data. Finally, we suggest that the sample sizes used in future studies are substantially increased to account for the significant variation in sporulation characteristics within *C. difficile* types.

The view of increased sporulation rates among hypervirulent *C. difficile* types is continually being spread at international meetings, as well as in print. Predictably, given the clinical importance of BI/NAP1/027 strains a substantial amount of time and funding is now being utilised to investigate the molecular and epidemiological basis of virulence in this type. Consequently, it is possible that current research directions associated with the sporulation mechanisms of *C. difficile* may be based on incorrect preliminary data. This review has highlighted the need for further investigations of *C. difficile* sporulation rates using a standard set of experimental procedures and larger sample sizes. Accordingly, we aim to evoke discussion within the *C. difficile* field of how sporulation should be measured with the aim of agreeing on a standard assay that can firmly interpret how emerging, hypervirulent *C. difficile* types differ from those types not associated with outbreaks of severe disease.

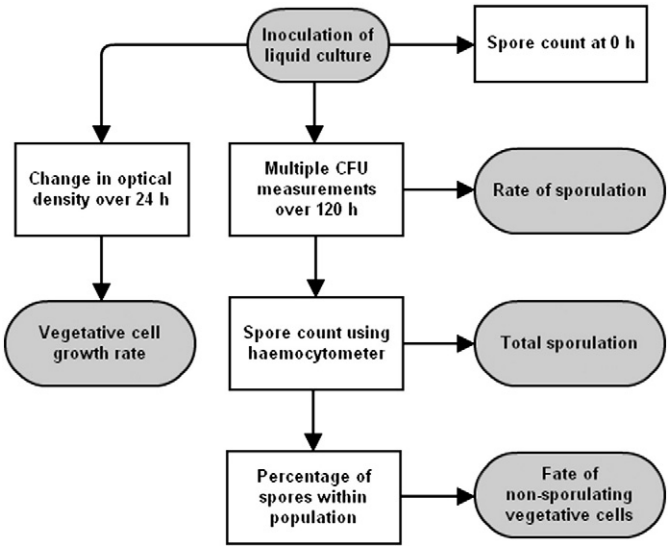

Fig. 1. A proposed experimental format for future investigations of *C. difficile* sporulation characteristics. This flowchart details the principles that, when applied together, can independently assess (i) vegetative cell growth rate; (ii) sporulation rate; (iii) total sporulation at a defined point; and (iv) the fate of vegetative cells that do not form spores. In addition, the suitable controls described in the text ensure that resultant data are not affected by technical errors.

Acknowledgements

Work in the authors' laboratory has received funding from the UK MRC (grant G0601176), the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 223585, and Morvus Technology Ltd.

We thank Stephen Cartman, John Heap and Daniela Heeg for helpful discussions and critical reading of the manuscript.

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